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DARPA ANTIBODY TECHNOLOGY PROGRAM STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF TWO MS2 SCFV ANTIBODIES PRODUCED BY THE UNIVERSITY OF TEXAS

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14. ABSTRACT:
Antibody selection for inclusion in a final assay format has relied on the performance of an antibody in an enzyme-linked immunosorbent assay with little regard to the quantification of the full spectrum of variables affecting antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV), Critical Reagents Program has instituted a quality program for the standardization of test methods to support comprehensive characterization and comparison of the physical and functional properties of antibody reagents within its repository. The development and standardization of antibody testing provides JPMO BSV with an invaluable platform for the provision of consistent, high-quality assays and reagents for current biodetection platforms and for the development and validation of future systems. This platform will be used to characterize and evaluate the MS2 recombinant antibody produced and supplied by the Defense Advanced Research Project Agency-funded investigator, the University of Texas (Austin, TX), for affinity and stability enhancements. The results of this study provide standardized parametric data on antibody properties and performance. They contribute to the development of a decisional analysis tool for expanding the confidence level of antibody-based reagent selection to optimize field operational and performance metrics for future detection and diagnostic platforms.

15. SUBJECT TERMS

Antibody	Biosurveillance	Antibody Technology Program (ATP)
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PREFACE

The work described in this report was supported by funding from the Defense Advanced Research Projects Agency. The work was started in October 2010 and completed in September 2012.

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1. INTRODUCTION

Current platforms for the detection and diagnosis of biothreat agent exposure depend on the use of antibodies to recognize and bind to specific antigens. To date, the selection of antibodies for inclusion in a final assay format has primarily relied on the performance of an antibody in an enzyme-linked immunosorbent assay (ELISA) with little regard for the quantification of the full spectrum of variables affecting antibody–antigen interactions. Members of the Joint Product Management Office for Biosurveillance (JPMO BSV), Critical Reagents Program (CRP) instituted a quality program for the standardization of test methods to fully characterize and compare the physical and functional properties of antibody reagents in its repository. The development and standardization of antibody testing provide JPMO BSV members with an invaluable platform for the provision of consistent, high-quality assays and reagents for existing biodetection platforms. In addition, they ensure the development and validation of future systems. This platform will be used to characterize the MS2 single-chain fragment variable (scFv) antibody, which has been modified and produced at the University of Texas (UT; Austin, TX) for the Defense Advanced Research Projects Agency (DARPA; Arlington, VA), Antibody Technology Program (ATP).

The DARPA ATP team focuses on developing technologies for enhancing the thermal stability and binding affinity of a given antibody. Functioning as an independent testing laboratory for this program, a team from the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) provided specific technical support on immune reagents and defined the government-supplied antibody–antigen pairs. The goal of this project was twofold: (a) select, develop, and standardize the methods for characterizing the de novo thermal and binding properties of select reagents to be used by DARPA-funded investigators and (b) use those methods to validate the changes in antibody thermal stability and binding affinity that were achieved by the DARPA investigators.

The antibody chosen for this project was the MS2 recombinant scFv that was produced at ECBC (*1*) and modified at UT. This antibody is used to detect an MS2 coat protein (MS2CP) that forms the capsid for the MS2 bacteriophage. The focus of the work highlighted in this report was the evaluation of the MS2 antibodies supplied by the DARPA-funded investigator, UT, for affinity and stability enhancements. The results of this study not only provide standardized parametric data on antibody properties and performance, but they also contribute to the development of a decisional analysis tool. This information will increase the confidence level during the selection of antibody-based reagents that will optimize the field operational and performance metrics of future detection and diagnostic platforms.

2. MATERIALS AND METHODS

2.1 MS2 scFv and MS2CP

The MS2 scFv antibody was produced at ECBC from a plasmid supplied by Ellen Goldman (U.S. Naval Research Laboratory, Bethesda, MD). The plasmid was designated Gv1, and the sequence was cloned into a pET-22b(+) plasmid (EMD Millipore, Billerica, MA). The protein was produced and eluted in 20 mM of sodium phosphate (pH 8.0), 0.5 M of sodium chloride, and 0.5 M of imidazole. Peak fractions were then collected and separated on a 16/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences, Pittsburgh, PA), and fractions that corresponded with a monomeric protein were collected and flash-frozen in liquid nitrogen. These fractions were provided, together with sequence data, to UT as baseline material.

The MS2CP was produced at ECBC from a pET-28a(+) plasmid (Novagen, Madison, WI). The MS2CP sequence was inserted with an amino acid substitution of an arginine at position 83 in a construct engineered by DNA2.0 Inc. (Menlo Park, CA). MS2CP was produced and eluted with 300 mM of imidazole in pH 7.4 phosphate-buffered saline (PBS; Sigma-Aldrich Company, St. Louis, MO). Peak fractions were collected, and buffer was exchanged into PBS (pH 7.4) using a 470 mL packed volume of Sephadex G-25 fine gel chromatography media (Amersham Biosciences Corporation, Piscataway, NJ). The fractions were provided to UT as antigen for the MS2 antibody.

2.2 UV-Visible Spectrophotometry

A NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Inc., Waltham, MA) was used to determine the MS2 scFv concentrations and the absorbance of light at 280 nm (A_{280}) for the samples supplied by UT. The A_{280} value was influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with the A_{280} value to determine an accurate concentration. The MS2 scFv concentrations were determined by dividing the average A_{280} value by 1.77, which is the extinction coefficient for a scFv. Each reading required a 2 µL sample, which was placed on the sample pedestal. The arm of the instrument was lowered, which created a liquid column between the top of the arm and the surface of the pedestal; the laser passed through this path length. The instrument was blanked using PBS, and readings were taken in triplicate. A positive control, bovine γ-globulin (BGG; Bio-Rad Laboratories, Hercules, CA), was tested to validate the instrument operation.

2.3 Electrophoresis

Molecular weight and purity data were collected using an Experion automated electrophoresis system (Bio-Rad). This system uses microfluidic technology to automate electrophoresis for protein analysis. The microfluidic chip, in conjunction with the Experion reagents, electrophoresis station, and software are designed to accomplish separation, staining, destaining, detection, and basic data analyses. The Experion Pro260 analysis kit is used with the Experion automated electrophoresis system. It uses engineered lower and upper internal alignment markers to provide clean baselines, accurate molecular weight sizing, and quantitative

protein analysis (*2*). In addition, the Pro260 analytical software is used to determine sample purity and calculate the percent mass of the separated proteins in a sample. For Experion analyses, each of the UT MS2 scFvs was standardized to a final concentration of 1 mg/mL and diluted in PBS. The BGG control and UT samples were then processed using the following validated procedure specified in the Pro260 analysis kit, rev. C (*3*):

- A Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells.
- The chip was primed on the priming station for 1 min at the medium (B) pressure setting.
- The priming process filled the fluidic channels with gel, which was used by the instrument to form a barrier between the samples during the run.
- A sample was reduced with dithiothrietol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before it was applied to the primed chip.
- The chip was then placed in the instrument, and the lid was closed, which lowered the sample needles into the wells.
- The instrument was operated using the Experion software; each chip took 30 min to complete.
- All samples were run in triplicate alongside one sample of the BGG control and the Pro260 ladder.
- All data analyses were performed using the Experion software.

2.4 Dynamic Light Scattering (DLS)

The DLS technique was used to show how the proteins behaved in solution. The DLS data indicated whether a protein was in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. Prediction algorithms within the software produce a range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the UT MS2 scFvs, along with the control bovine serum albumin (Sigma-Aldrich), were placed in a quartz 384-well plate (Wyatt Technology Corporation, Santa Barbara, CA) and centrifuged for 2 min at 239 ×*g* to remove trapped air bubbles from the samples. Mineral oil (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation. The plate was placed in a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s at 25 °C. Values were averaged to provide measurements of the polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample using Dynamics software (Wyatt Technology). The results of three wells were averaged and reported.

2.5 Differential Scanning Calorimetry (DSC)

The DSC technique was used to obtain a quantitative melting temperature (T_m) for each of the UT MS2 scFv proteins. T_m is used to predict the results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m > 70 °C predicts that the percent

of antibody activity after the thermal stress test will remain above 50%. A T_m < 70 °C predicts at least a 50% decrease in antibody activity after the thermal stress test. For the DSC experiments, samples were diluted to 0.5 mg/mL and dialyzed overnight in PBS (pH 7.4). The samples were degassed for 5 min before analysis and injected into the sample cell of a MicroCal VP-DSC microcalorimeter (Malvern Instruments, Malvern, Worcestershire, UK). Dialysis buffer was added to the reference cell of the calorimeter, and a buffer scan was used as the baseline for all the experiments. The samples (in duplicate) were scanned from 15 to 100 °C at a rate of 60 °C/h. The transition midpoint of the protein was determined by data analysis using Origin 7.0 software (MicroCal).

2.6 Thermal Stress Test

Before heat was applied, samples were diluted to 1 mg/mL to negate protective effects due to concentration (*2*). The UT thermally stabilized antibody (KP1) was diluted to 1 mg/mL in 1× PBS and divided into five tubes. One aliquot was kept on ice for the duration of the experiment and was marked as time 0. The remaining four aliquots were heated to 70 °C on a calibrated heat block for 15, 30, 45, and 60 min each. After each time point, the corresponding aliquot was removed and placed in an ice bath. These samples were then tested for activity.

2.7 ELISA

ELISAs were performed in triplicate using standard techniques. After the thermal stress test, each sample was diluted to 1 µg/mL in PBS and used to coat one row each of three Nunc MaxiSorp 96-well plates (Thermo Scientific) that were subsequently incubated at 4 °C overnight. In the morning, each plate was washed in 1× wash buffer (KPL, Inc., Gaithersburg, MD) using a standard wash protocol on an AquaMax 200 plate washer (Molecular Devices, Sunnyvale, CA). The plate was blocked with 1× milk diluent block (MDB; KPL) for 30 min at 37 °C. The plate was washed, and PBS with 0.05% Tween 20 (PBS-T; Sigma-Aldrich) was applied to the plate so that each well received 100 µL of PBS-T. MS2CP was diluted in PBS-T to 2 µg/mL, and 100 µL of the PBS-T was applied to the first well of each row. A twofold serial dilution was performed across the plate, and it was incubated for 1 h at 37 °C. After the plate was washed, mouse anti-MS2 (supplied by the JPMO BSV CRP) was diluted to 5 µg/mL in 1× MDB, and 100 µL of this dilution was added to each well. The plate was incubated at 37 °C for 1 h and then washed. Goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP; KPL) was diluted to 0.2 µg/mL in 1× MDB, and 100 µL of this dilution was added to each well. The plate was incubated at 37 °C for 30 min. After the plate was washed, 100 µL of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) at room temperature and one component HRP substrate (KPL) were added to each well. After 20 min of exposure at 37 °C, the optical density at the 405 nm light wavelength was determined using a Synergy H4 hybrid multi-mode microplate reader (BioTek, Winooski, VT). Data analysis was performed using Prism software (GraphPad Software, La Jolla, CA).

2.8 SPR Methodology

SPR methodology is used to determine the kinetic parameters of antibody–antigen interactions and to rapidly monitor biomolecular interactions through excitation of surface plasmons. Polarized light is shone through a prism on a sensor chip with a thin metal film coating, which reflects the light by acting as a mirror. If the angle of light shone through the prism is changed, and the intensity of the reflected light is monitored, differences in intensity can be recorded. Although the refractive index at the prism side of the chip does not change, the refractive index in the immediate vicinity of the metal surface will change when accumulated mass (bound proteins) adsorbs on the surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this SPR angle shift provides information on the protein adsorption kinetics on the surface. The software can then provide an accurate analysis of the association (k_a) and dissociation (k_d) rate constants for the antibody interactions, as well as calculate the overall antibody–antigen affinity constant (K_D).

2.8.1 Thermostability Testing Using SPR

A Biacore T200 system (GE Healthcare) and standard amine coupling chemistry were used to tether 6500 response units (RUs) of MS2CP to one flow cell of a Biacore CM5 sensor chip. After a thermal stress test was performed, samples were centrifuged at 2000 ×*g* and 5 °C for 5 min. The analyte was run at 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0 unheated UT MS2 scFv samples (KP1 or KP3) at 400, 350, 300, 250, 200, 150, 100, and 50 nM and by plotting the maximum analyte-binding RU surface capacity of each. Unheated and heated samples were then diluted to 1:90 and 1:180, respectively, so that the time 0 control points fell on the linear calibration curve. All samples were run in triplicate. The chip's surface was regenerated with an 18 s injection of 0.85% of phosphoric acid at a flow rate of 30 µL/min. Data was collected using the Biacore concentration analysis software, and the active concentration of heated sample was recorded. The running buffer used for this experiment was Biacore HBS-EP 1× buffer (GE Healthcare Life Sciences).

2.8.2 Kinetic Analysis Using SPR

A ProteOn XPR36 SPR system (Bio-Rad), PBS-T running buffer, and standard amine coupling chemistry were used to tether 200 RU of MS2CP to a GLC sensor chip (Bio-Rad). UT MS2 scFv samples (KP1or KP3) were injected across the chip's surface for 120 s at a flow rate of 100 µL/min with a 600 s dissociation at 5 nM, 1.67 nM, 560 pM, 190 pM, and 60 pM. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 100 µL/min. Data was analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 Spectrophotometry Results

Both of the UT-produced MS2 scFvs were read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings are shown in Table 1.

Table 1. NanoDrop A_{280} Readings

Replicate No.	KP1 (mg/mL)	KP3 (mg/mL)
1	3.142	1.901
2	3.199	1.843
3	3.151	1.921

For each antibody, these three numbers were averaged and divided by the extinction coefficient of 1.77. The final concentrations were determined to be 1.79 mg/mL for the thermally stabilized KP1 and 1.07 mg/mL for the affinity-matured KP3.

3.2 Electrophoresis Results

The molecular weights of the UT-produced MS2 scFvs were determined using the Experion Pro260 analysis kit (Figure 1). The thick bands in the second and fourth lanes correspond to the MS2 scFv antibodies KP1 and KP3, respectively. According to the Experion software, KP1 was 90.1% pure and weighed 32.6 kDa, and KP3 was 92.1% pure and weighed 33.9 kDa.

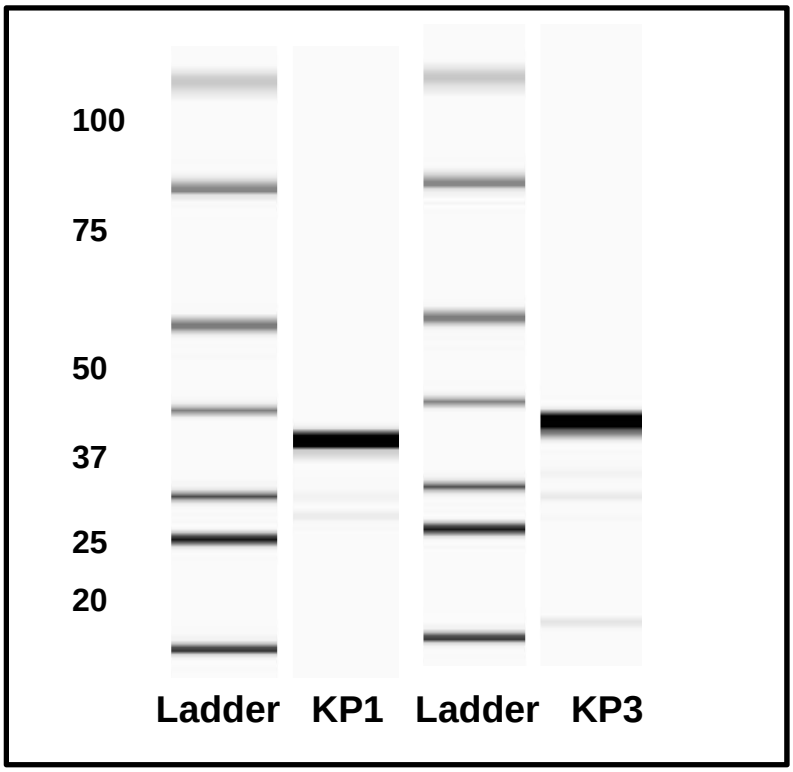

Figure 1. Molecular weight and purity: digital gel of UT MS2 scFvs produced using the Experion Pro260 analysis kit. The thick bands in the second and fourth lanes correspond to the MS2 scFv antibodies KP1 and KP3, respectively.

3.3 DLS Results

Both of the UT-produced MS2 scFvs were analyzed in triplicate using the DynaPro plate reader. The KP1 radius was determined to be 2.5 nm with a polydispersity of 9.9% (Table 2), whereas the KP3 radius was determined to be 2.6 nm with a polydispersity of 10.8%. Figure 2 contains representative correlation and regularization graphs for each of the MS2 scFvs. The correlation graphs (Figures 2A and 3A) depict a sigmoidal curve indicative of a valid size distribution. The regularization graphs (Figures 2B and 3B) illustrate the monodispersity found in both samples. Table 2 shows the raw data produced for each replicate. Because 100% of the mass displayed favorable polydispersity and hydrodynamic radius, both of these sample preparations were considered to be monodisperse.

Table 2. Features of MS2 scFvs in Solution

Sample	Replicate	Radius (nm)	Polydispersity (%)
KP1	1	2.3	6.3
	2	2.6	11.8
	3	2.5	11.2
	4	2.6	14.0
	5	2.4	6.3
	Average	2.5	9.9
KP3	1	2.7	11.1
	2	2.5	10.9
	3	2.5	10.3
	Average	2.6	10.8

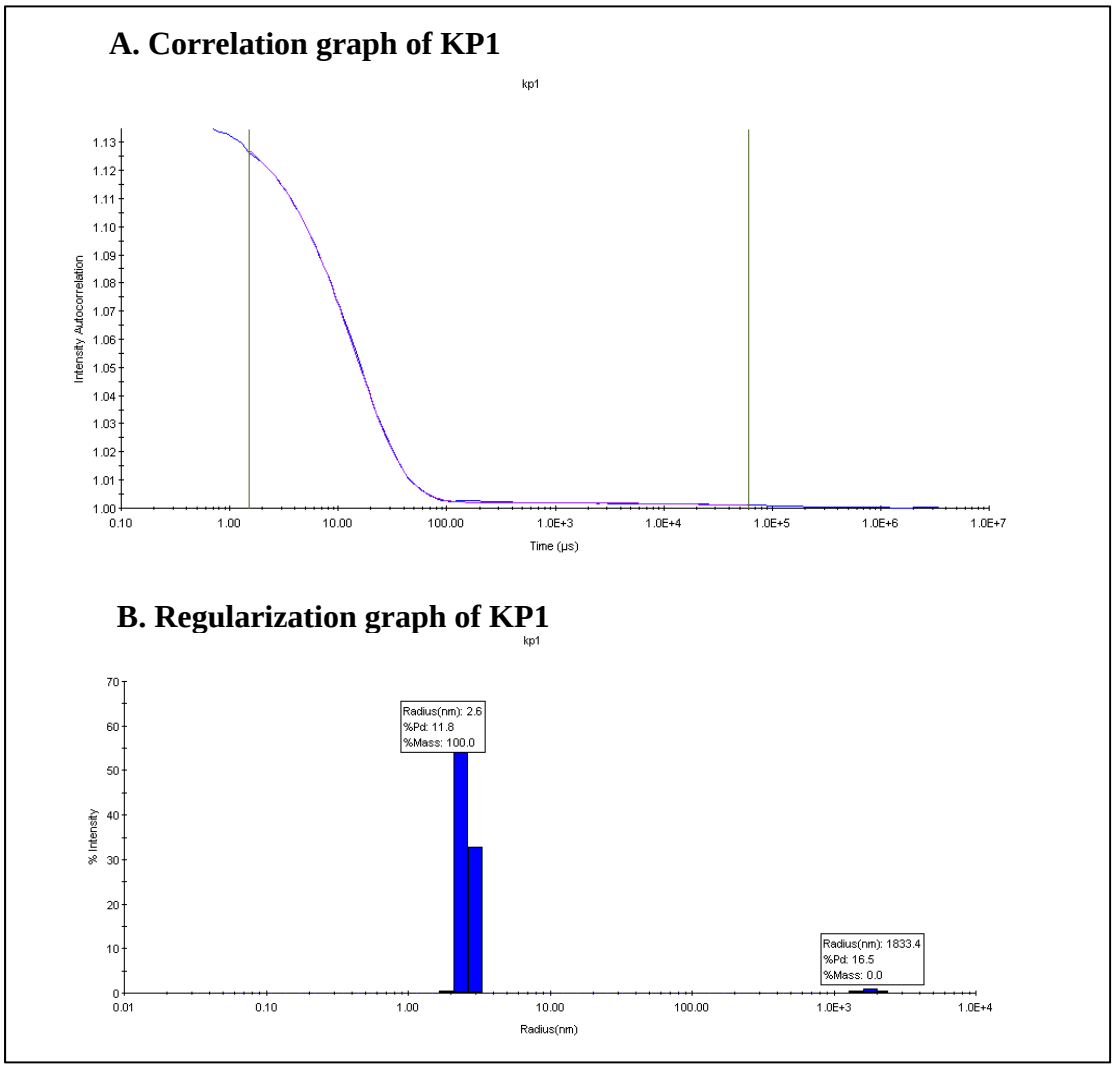

Figure 2. Radius and polydispersity representation of the UT thermostable scFv KP1. **(A)** Correlation and (**B)** regularization graphs.

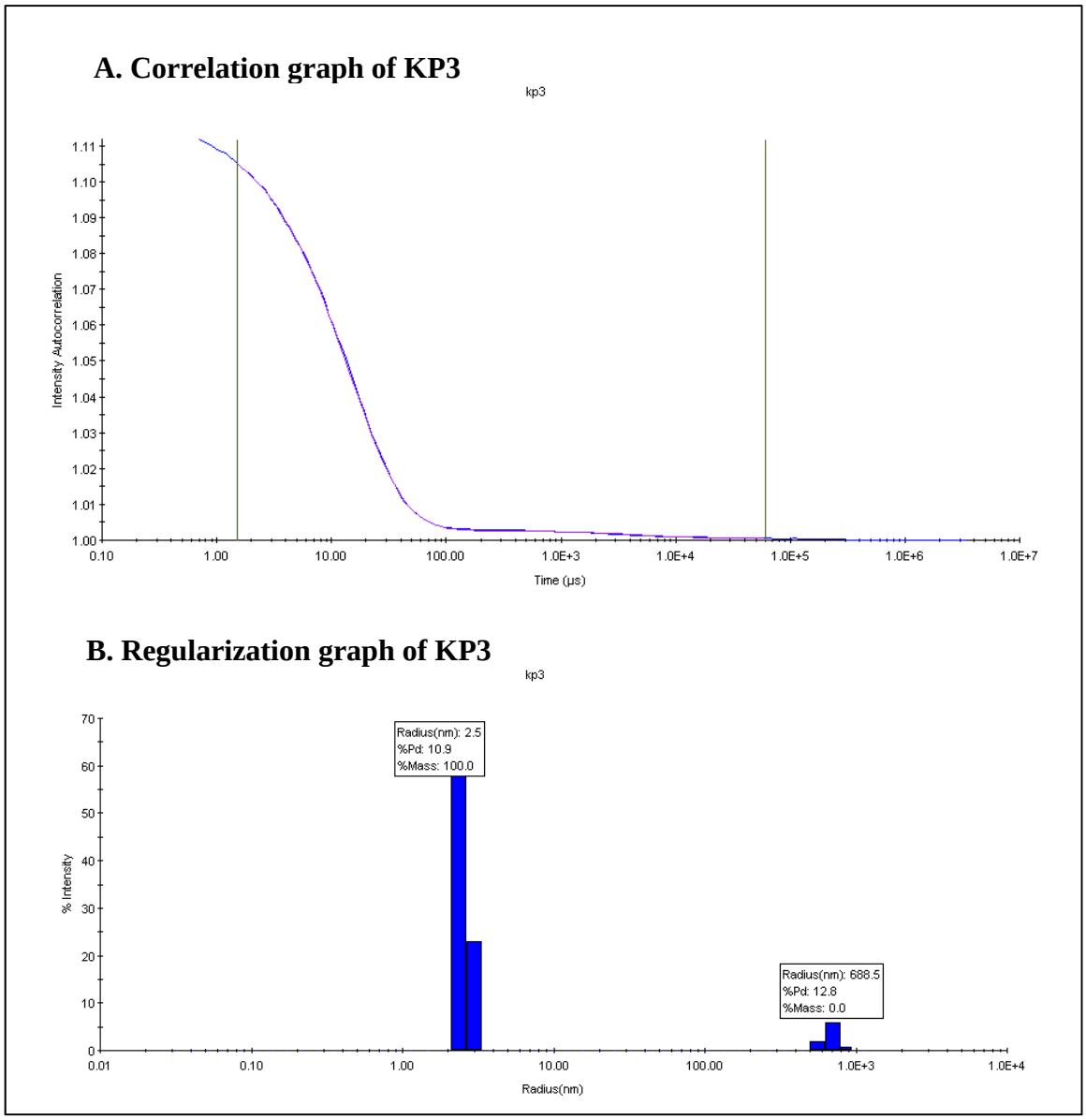

Figure 3. Radius and polydispersity representation of the UT affinity-matured scFv KP3. **(A)** Correlation and (**B)** regularization graphs.

3.4 DSC Results

The UT MS2 scFvs were read in duplicate on the MicroCal VP-DSC. The peak T_m values were as follows:

- KP1 Replicate 1: 68.38 °C,
- KP1 Replicate 2: 68.25 °C,
- KP3 Replicate 1: 67.23 °C, and
- KP3 Replicate 2: 67.38 °C.

The final T_m value for KP1 was determined to be 68.32 °C (Figure 4), and the T_m value for KP3 was determined to be 67.30 °C (Figure 5).

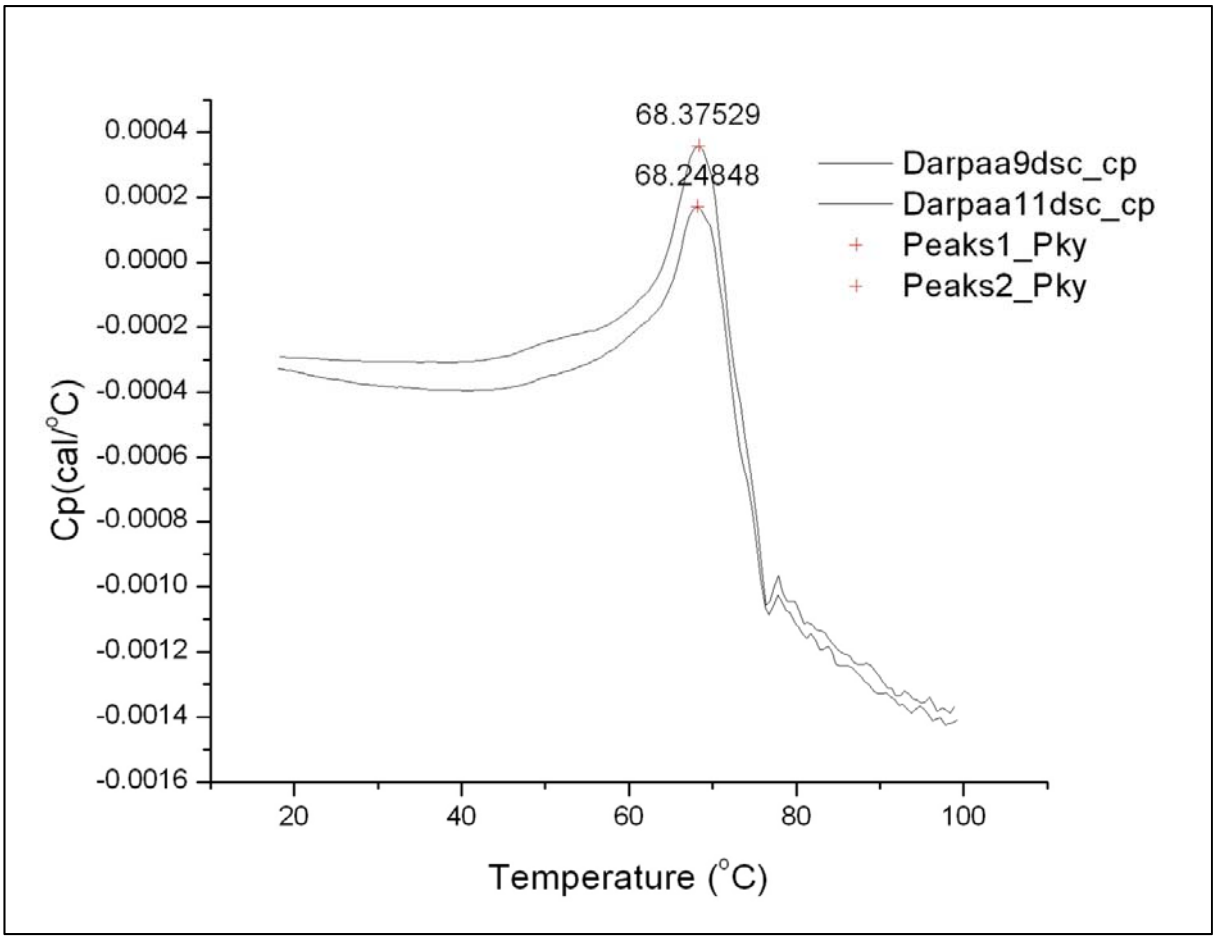

Figure 4. Transition midpoint curve for the thermo-enhanced UT MS2 scFv KP1. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the Peak Find function in the Origin 7.0 software. The transition midpoint was calculated to be 68.3 °C for KP1.

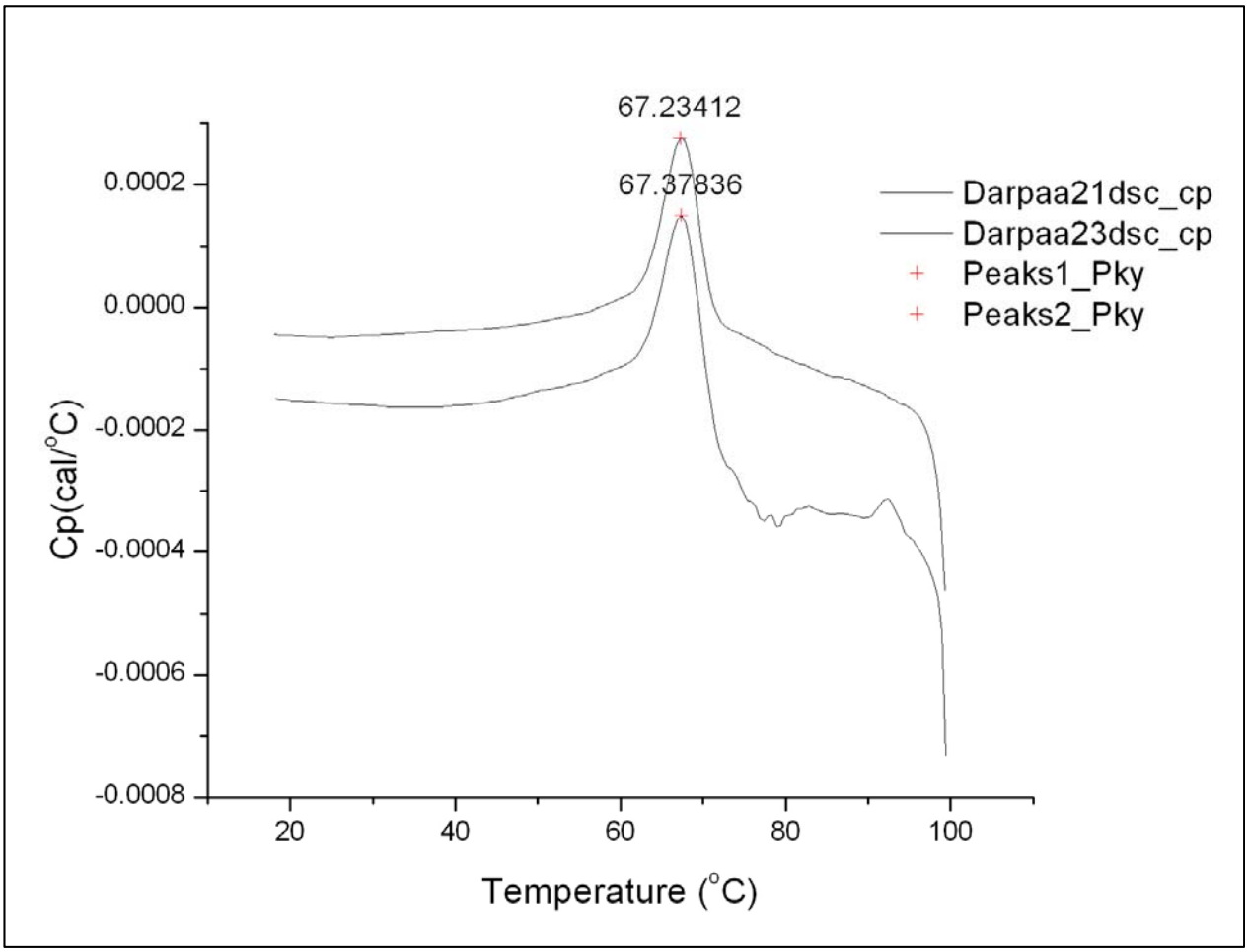

Figure 5. Transition midpoint curve for affinity-matured UT MS2 scFv KP3. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the Peak Find function in the Origin 7.0 software. The transition midpoint was calculated to be 67.3 °C for KP3.

3.5 ELISA Results

ELISA assays were used to test the functional interaction of the antibody and antigen after thermal stress at 70 °C. The ELISA data (Figure 6) show that when the UT scFv KP1 was heated to 70 °C, in a manner similar to the government-supplied MS2 scFv, it retained some of its activity at 70 °C for 30 min, but had no activity after 40 min. The curves in Figure 6A show antibody activity for different time points at 70 °C as a function of the concentration of the antigen supplied. The area under the curve for each of the different time points at 70 °C was calculated, averaged, and graphed to depict how the MS2 scFv reacted over time to thermal stress. The graph in Figure 6B illustrates that little of the MS2 scFv remained functionally capable of binding to antigen after exposure to 70 °C, like the original scFv, which lost activity within 15 min of heating.

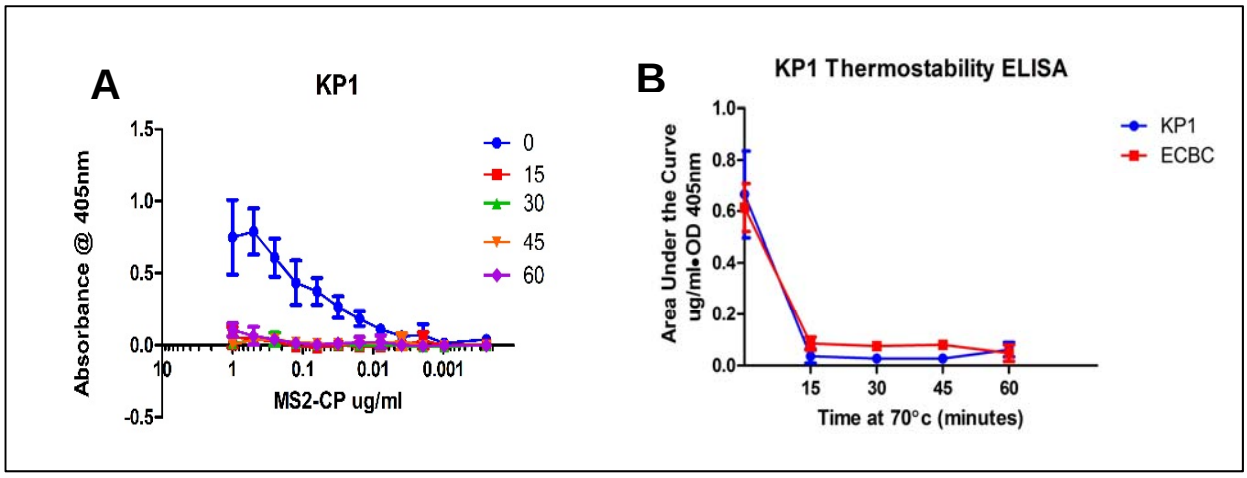

Figure 6. Thermostability of UT MS2 scFv KP1 using ELISA. **(A)** Activity of KP1 as a function of antigen concentration after thermal stress; the different curves are represented on the legend by the number of minutes the sample was held at 70 °C and **(B)** area under the curve analysis depicting the effect of thermal stress.

3.6 SPR Results

3.6.1 Thermostability Testing Results Using SPR

After the antibody–antigen complex was heated to 70 °C for variable time periods, SPR was used to assess the functional binding between the UT MS2 scFv (KP1) and the antigen. Five tubes of 1 mg/mL MS2 scFv were prepared and heated to 70 °C for the following time periods: 15, 30, 45, and 60 min, before they were quenched on ice. A Biacore T200 system was used to compare the activity of each sample with a calibration curve for unheated sample. The percentage activity of the heated samples was plotted over time (Figure 7). The results indicated that the scFv retained some activity at 30 min, whereas the activity of the scFv dropped off completely within the first 15 min at 70 °C.

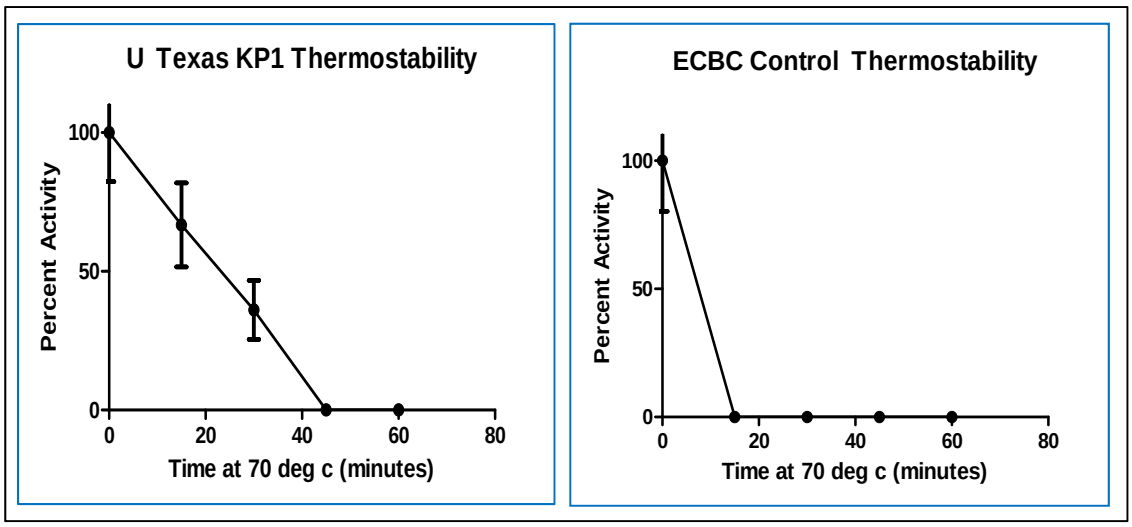

Figure 7. (Left) Thermostability of the UT MS2 scFv (KP1) and (right) comparison with the ECBC MS2 scFv by SPR. The UT antibody maintained some of its activity after heating to 70 °C for 30 min, compared with the original ECBC MS2 scFv antibody, which lost all ability to recognize the MS2CP target within 15 min of heating.

3.6.2 Kinetic Analysis Results Using SPR

Kinetic analysis of the affinity-enhanced UT MS2 scFv (KP3) binding to the MS2CP antigen was performed as a direct-binding SPR experiment on the Proteon XPR36 (Figure 8). Data were normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using the Bio-Rad Proteon XPR36 software. The K_D was determined to be 32.9 pM. Similar experiments that were performed using the original ECBC MS2 scFv are presented in Figure 9. The K_D of the original scFv was determined to be 8.66 nM; thus, UT provided an antibody that exceeded the 100-fold improvement threshold. Finally, kinetics analysis was performed on the thermostable-enhanced UT MS2 scFv (KP1), and the results are presented in Figure 10. The K_D was determined to be 262 pM.

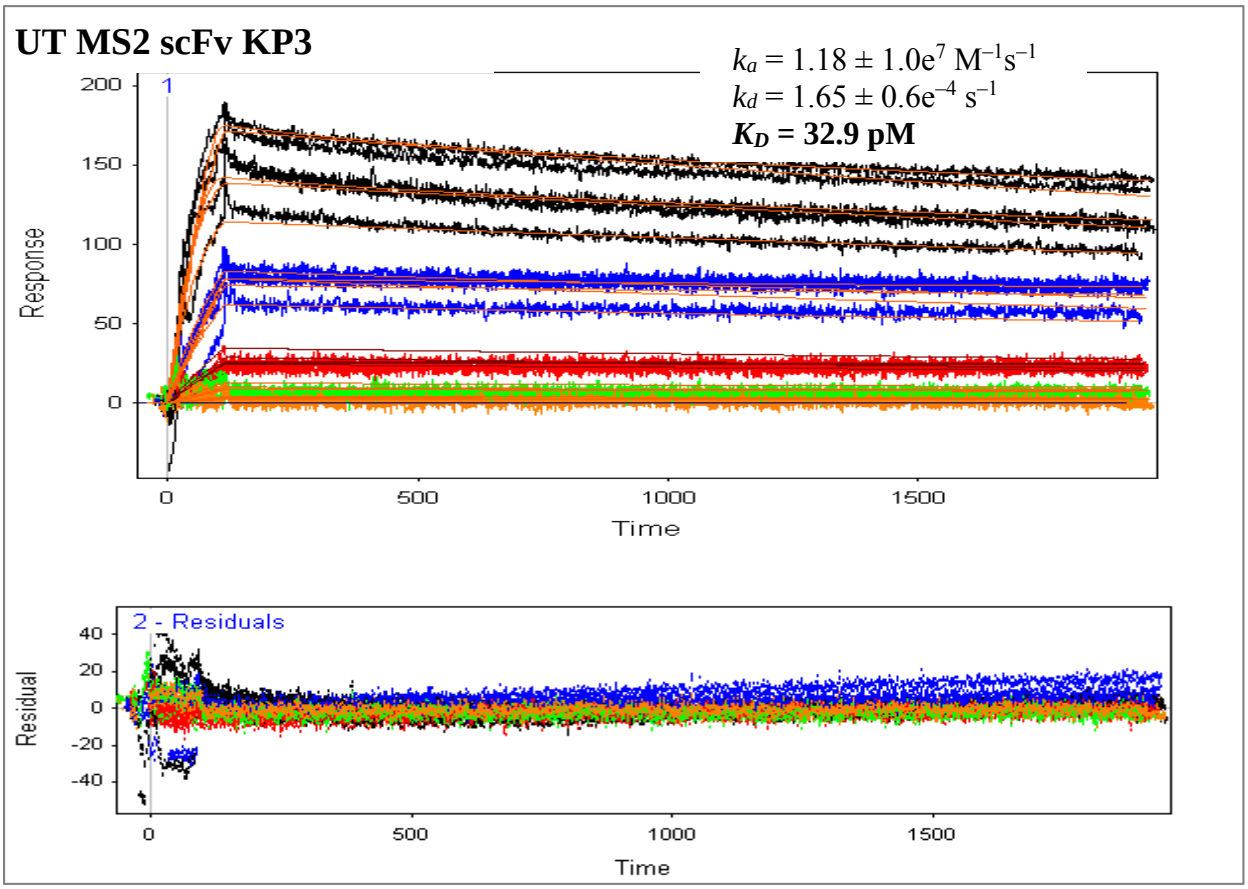

Figure 8. Comparison of the kinetic fits with residuals of the MS2 scFv KP3 antibodies, determined using a Proteon XPR36 system (kinetics of UT affinity-enhanced MS2 scFv [KP3; 32.9 pM]).

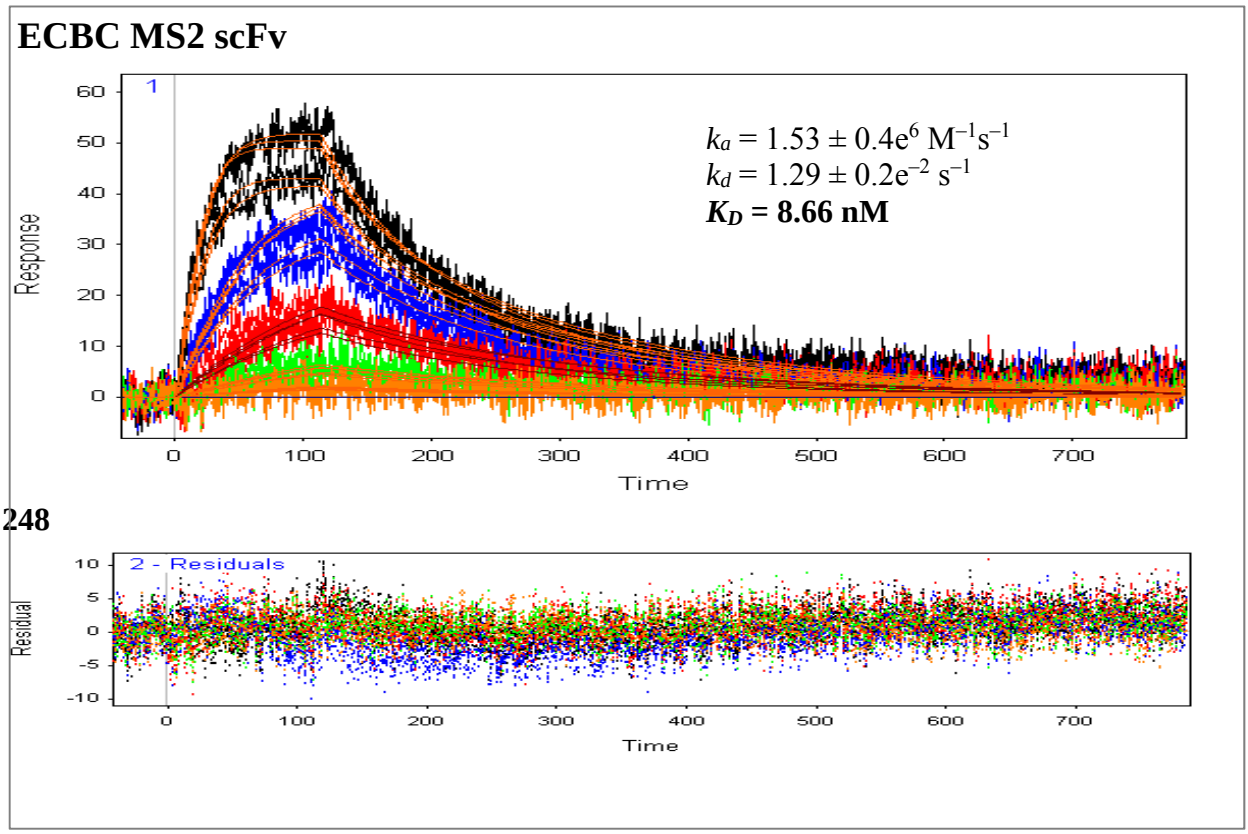

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Figure 9. Comparison of the kinetic fits with residuals of the ECBC MS2 scFv antibodies, determined using a Proteon XPR36 system (kinetics of original MS2 scFv [15.5 nM]).

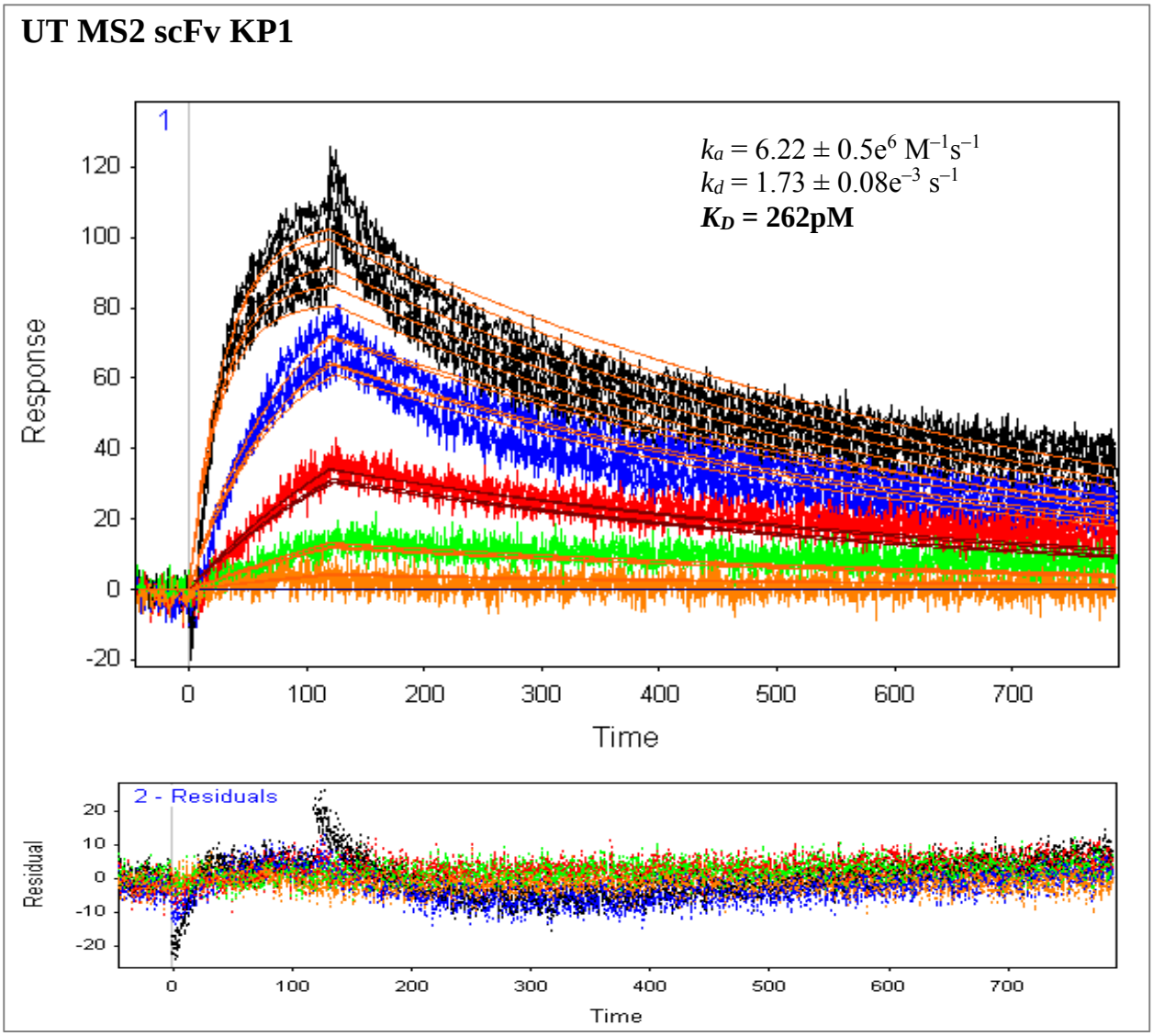

Figure 10. Comparison of the kinetic fits with residuals of the UT MS2 scFv KP1 antibodies, determined using a Proteon XPR36 system (kinetics of UT thermo-enhanced MS2 scFv [KP1; 262 pM]).

4. DISCUSSION

This study established and standardized the parametric tests for performance on the MS2 scFv antibody. This antibody was selected by the DARPA ATP as the initial substrate for demonstrating molecular schemes for improving the thermal stability and affinity of its target antigen. The test bed that was developed was used to define the physical and functional properties of the reference MS2 scFv antibody and to establish the baseline for subsequent testing of the engineered antibodies submitted by the ATP scientists. A snapshot of the physical characteristics of the MS2 scFvs was obtained using the NanoDrop, Experion, and DLS measurement platforms. Measurements of the MS2 scFv functional characteristics for assessing

the effects of molecular engineering on thermal stability and affinity were obtained using the DSC, ELISA, and SPR analytic platforms.

An accurate assessment of protein concentration is critically important for all of the test procedures described in this report. We applied the standard technique of spectrophotometry using the NanoDrop ND-1000 system. This instrument was employed to provide the A_{280} value of the sample, which was influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with the A_{280} value to determine an accurate concentration.

After concentration was determined using spectrophotometry with the NanoDrop system, molecular weight and purity data were collected with the Experion automated electrophoresis system. This system employs microfluidic technology to automate electrophoresis for protein analysis. The results of Experion analysis of the MS2 scFv protein fell within the acceptable range of purity for use in assay development, and the molecular weight determined by the software (shown in Figure 1) was typical for an scFv.

DLS was used in conjunction with the Experion and NanoDrop systems to illustrate how the protein behaved in solution. DLS data indicate the physical state and potential aggregation of a protein in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. The DLS data established whether the MS2 scFvs provided by UT were monomeric and monodisperse. Less than 1% of the sample mass appeared to aggregate in solution (Figures 2 and 3). To ensure that all testing was consistent and to mitigate the exacerbating effect of freeze-thawing on future sample aggregation, the UT MS2 scFvs were aliquoted into single-use vials and centrifuged before use.

In the next round of testing, the thermostability of the UT MS2 scFvs were evaluated using the DSC, ELISA, and SPR systems. DSC was used to obtain a quantitative T_m value for the MS2 scFvs. The T_m value should predict the results of ELISA and SPR thermostability testing. $T_m > 70$ °C predicts that the percent activity of the MS2 scFvs after thermal stress should remain above 50%. $T_m < 70$ °C predicts at least a 50% decrease in MS2 scFv activity after thermal stress. The UT MS2 scFv antibody that was described in this study and optimized for thermostability (KP1) exhibited $T_m = 68.6$ °C (Figure 4), compared with $T_m = 67.5$ °C shown in the original MS2 scFv study (*4*). Therefore, it was expected that heating this sample above 70 °C would cause the sample to unfold and lose at least 50% of its activity when evaluated using the ELISA and SPR systems.

The results of the thermal stress test demonstrated that the UT MS2 scFv remained active for less than 45 min of heating at 70 °C. The ELISA and SPR data confirmed that the UT MS2 scFv (KP1) was unable to bind the MS2CP after 60 min of heating. This result was similar to that of the original MS2 scFv reference antibody, which was unable to bind the MS2CP after only 15 min of heating at 70 °C (Figures 6 and 7).

SPR was also used to obtain a kinetic analysis of the affinity-enhanced UT MS2 scFv (KP3) binding to its target antigen MS2CP to compare binding parameters with the original antibody. Kinetic data for KP3 MS2 scFv binding to the MS2CP was obtained using the Proteon

XPR36 SPR platform, which yielded a K_D of 32.9 pM, whereas the original MS2 scFv yielded a K_D of 8.66 nM (Figures 8–10). The affinity K_Ds clearly show that UT exceeded the 100-fold improvement requested by DARPA.

5. CONCLUSIONS

Members of the DARPA ATP seek to establish methods for rapidly engineering a given antibody reagent to exhibit physical and functional properties that far exceed those of its native state and thereby, expand user confidence in fielding antibody-based detection and diagnostic platforms in environments or operational scenarios in which currently available reagents exhibit degradation or interference. By optimizing the thermal stability and binding affinity of an antibody for its biological target, the DARPA ATP will develop antibody reagents that can reliably function in harsh environmental conditions and increase the sensitivity of sensor platforms to detect lower levels of threat agents.

This report documents the testing of an improved thermostable antibody (KP1) and an affinity-improved antibody (KP3), which were produced by UT. This study evaluated the physical and functional characteristics of both scFvs in the ECBC testing pipeline. The results were compared to the baseline characteristics of the physical properties of the original antibody (i.e., concentration, molecular weight, purity, and state of aggregation in solution and functional measures such as binding affinity and thermal stability). Both antibodies supplied by UT exhibited enhanced thermal stability and affinity for binding to the MS2CP antigen; however, the thermal enhancements of KP1 did not meet the thermostability requirement of the project.

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ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance of light at 280 nm
ATP	Antibody Technology Program
BGG	bovine γ-globulin
CRP	Critical Reagents Program
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DSC	differential scanning calorimetry
ECBC	U.S. Army Edgewood Chemical Biological Center
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
JPMO BSV	Joint Product Management Office for Biosurveillance
k_a	association rate constant
k_d	dissociation rate constant
K_D	affinity constant
MDB	milk diluent block
MS2CP	MS2 coat protein
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% Tween 20
RU	response unit
scFv	single-chain fragment variable
SPR	surface plasmon resonance
T_m	melting temperature
UT	University of Texas

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